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Award Number: DAMD17-01-1-0659

TITLE: Agonist-Occupied PRA Represses PRB via Interactions with Coactivators or Corepressors

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REPORT DATE: July 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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20030109 078

REPORT DOCUMENTATION PAGE

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE July 2002	3. REPORT TYPE AND DATES COVERED Final (1 Jul 01 – 30 Jun 02)

4. TITLE AND SUBTITLE
Agonist-Occupied PRA Represses PRB via Interactions with
Coactivators or Corepressors

5. FUNDING NUMBERS
DAMD17-01-1-0659

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**8. PERFORMING ORGANIZATION
REPORT NUMBER**

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSORING / MONITORING
AGENCY REPORT NUMBER**

11. SUPPLEMENTARY NOTES
Report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Hypothesis: the ability of agonist-occupied PRA (progesterone receptor A form) to act as a dominant negative inhibitor of many other receptors including progesterone receptor B form (PRB),) is due to PRA's capacity to sequester away a common coactivator or recruit a common corepressor. Likely candidates for interaction include the coactivators SRC-1 and TIF-2; corepressor candidates include NCoR and SMRT. Color variants of green fluorescent protein (GFP) were utilized for tagging PRA, PRB, SRC-1, TIF-2, NCoR, and SMRT. GFP-tagged proteins were then used in colocalization and confocal microscopy interaction studies. The purpose of this work was to find differential interactions between PRA and PRB and the above listed coactivators and corepressors. An extension of this project is cellular kinetic studies of drug-receptor complexes in living cells. The hypothesis of this part of the project was to correlate the rate of import into the nucleus of drug-occupied PRB with the dose of drug and hence the transcriptional activity of the drug. The global scope of this project was to better understand the precise roles of PRA and PRB with other cellular factors, as this is critical for finding better therapeutic targets and improved treatments for steroid-associated breast cancers.

14. SUBJECT TERMS PRB, PRA, Corepressor, breast cancer			15. NUMBER OF PAGES 9
			16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

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INTRODUCTION

Our original hypothesis intended to show that the ability of agonist-occupied PRA (progesterone receptor A form) to act as a dominant negative inhibitor of many other receptors including progesterone receptor B form (PRB),) is due to PRA's capacity to sequester away a common coactivator or recruit a common corepressor. Likely candidates for interaction include the coactivators SRC-1 and TIF-2; corepressor candidates include NCoR and SMRT. Color variants of green fluorescent protein (GFP) were utilized for tagging PRA, PRB, SRC-1, TIF-2, NCoR, and SMRT. GFP-tagged proteins were then used in colocalization and confocal microscopy interaction studies. The purpose of this work was to find differential interactions between PRA and PRB and the above listed coactivators and corepressors. An extension of this project is cellular kinetic studies of drug-receptor complexes in living cells. The hypothesis of this part of the project was to correlate the rate of import into the nucleus of drug-occupied PRB with the dose of drug and hence the transcriptional activity of the drug. The global scope of this project was to better understand the precise roles of PRA and PRB with other cellular factors, as this is critical for finding better therapeutic targets and improved treatments for steroid-associated breast cancers.

BODY

Plasmid constructions

Plasmids encoding coactivators (SRC-1 and TIF-2) and corepressors (NCoR and SMRT) were obtained from outside sources listed below. Plasmids pEYFP-SRC-1A, pEYFP-GRIP (GRIP is also known as TIF-2), pECFP-NCoR, pECFP-SMRT, pECFP-PRA, pEYFP-PRA, pECFP-PRB, and pEYFP-PRB were constructed (subcloned) as follows:

For pEYFP-SRC-1A: pCR3.1 hSRC-1A was a gift from Bert W. O'Malley. Department of Cell Biology, Baylor College of Medicine, Houston, TX. PCR amplification of the YFP sequence from pEYFP-C1 using the primers 5' CAT GGT ACC ATG GTG AGC AAG GGC GAG GA and 3' CTG CAG AAC CAC CAC ACT GGA CTT GTA CAG CTC GTC CAT GC was performed to create a 5' *Kpn I* site and a 3' *Bst XI* site used for subcloning into pCR 3.1-hSRC-1a vector, which was digested with the same enzymes.

For pEYFP-GRIP: pEGFP-C2-GRIP/fl was a gift from Gordon Hager, Laboratory of Receptor Biology and Gene Expression, NIH, Bethesda, MD. The EYFP-GRIP plasmid was generated by sequential digestion of pEGFP-C2-GRIP/fl with *Age I* and then *EcoR I* to get the desired fragment. The 4.4kb fragment was ligated with vector pEYFP-C1, which was digested with *EcoR I* to create pEYFP-GRIP.

For pECFP-NCoR: pBKS-NCoR was a gift from Dr Christopher K. Glass, Department of Cellular & Molecular Medicine, Department of Medicine, University of California, San Diego. The plasmid ECFP-NCoR was constructed as follows: pECFP-*Not I* was generated by designing two oligomers 5' CCG GAC <u>TCG CGG CCG CGA</u> GAT CTC GAG CTC AAG CTT CG 3' and 5' AAT CGA AGC TTG AGC TCG AG ATC TCG CGG CCG CGA GT 3' containing a *Not I* restriction enzyme site. The

annealed oligomer was inserted into pECFP-C1 vector which was digested with *BspE I* and *EcoR I*. The plasmid PBKS-NCoR was digested with *Not I* and *Sal I*, and religated with vector pECFP-Not1 which was digested with the same restriction enzymes.

For pECFP-SMRT: pCMX-mSMRTα-FL was a gift from Ronald M. Evans, Gene Expression Laboratory, Howard Hughes Medical Institute, The Salk Institute for Biological Studies, La Jolla, CA. Plasmid CFP-SMRT construction: Two oligomers were designed: 5'CCG GAA CTC AGA TCT CGA GCT CAA GCT TCG ATA TCT GCA G 3' and 5' TCG ACT GCA GAT ATC GAA GCT TGA GCT CGA GAT CTG AGT T 3' which contain *EcoRV* restriction enzyme sites, in frame with SMRT. The annealed oligomer was inserted into an EcoRV-cut pECFP-C1 vector to generate plasmid pECFP-EcoRV. The plasmid pCMX-mSMRTa-FL was digested with *EcoRV* and religated with vector pECFP-EcoRV which was digested with the same enzyme.

For pECFP-PRB, pECFP-PRA, pEYFP-PRB, pEYFP-PRA: ECFP or EYFP-C1 were digested with *BspE I* and *ASE I*, and the CFP or YFP band collected. Each was religated separately into GFP-PRA or B vector digested with the same enzymes.

Transfections

Each plasmid (pEYFP-SRC-1A, pEYFP-GRIP, pECFP-NCoR, pECFP-SMRT, pECFP-PRA, pEYFP-PRA, pECFP-PRB, and pEYFP-PRB) was initially individually transfected into 1471.1 cells (mouse adenocarcinoma cell line that does not express either form of PR) by electroporation using previously described methods (Lim, Baumann et al. 1999). All plasmids were found to localize in cells as expected as observed under fluorescence microscopy. The nuclear coactivators pEYFP-SRC-1A and pEYFP-GRIP, and nuclear corepressors pECFP-NCoR and pECFP-SMRT all were found to localize in the nucleus of cells, as anticipated. The CFP- and YFP-tagged progesterone A receptor (pECFP-PRA and pEYFP-PRA) localized mainly in the nucleus of cells, while pECFP-PRB and pEYFP-PRB constructs were both nuclear and cytoplasmic, both as expected (Lim, Baumann et al. 1999). Photobleaching of CFP constructs was more pronounced than for YFP (and GFP) constructs.

Microscopy

FRET (fluorescence energy resonance transfer) studies (in our original proposal) are currently in progress. FRET can be used to detect *in vivo* protein-protein interactions, such as those that occur between PRB/A and a coactivator or corepressor. Energy transfer between proteins tagged with YFP and CFP occurs if the interacting proteins are within 50-100 angstroms of each other. As proof of concept, we are trying to detect an interaction between PRB and SRC-1. The plasmids pCFP-PRB and pYFP-hSRC-1a were transfected into 1471.1 cells; 24 hours later the cells were induced with the synthetic progestin R5020 (75nM) for 4 hours. The cells were fixed with 3% paraformaldehyde, then observed under the Zeiss LSM-510 confocal microscope. The cells are excited at the CFP (donor) wavelength and observed at the YFP (acceptor) emission wavelength (YFP excitation 517nm, emission 528nm; CFP excitation 436nm, emission 488nm). If the image is detected at the YFP wavelength, FRET has occurred (after correcting for crosstalk between channels). In figure 1 (not background subtracted yet), CFP is shown in cyan, YFP in red, and FRET in yellow. From this exciting preliminary study, it appears that the interaction between PRB and SRC-1 can be detected using FRET.

During this time, we found a paper by Giagrande et al. that was very similar to the goals of this project. From the abstract of this paper (Giangrande, Kimbrel et al. 2000): "Specifically, it was determined that hPRA has a higher affinity for the corepressor SMRT than hPRB... Together, these data indicate that the ability of hPRA to transrepress steroid hormone receptor transcriptional activity and its inability to activate progesterone-responsive promoters occur by distinct mechanisms. To this effect, we observed that hPRA, unlike hPRB, was unable to efficiently recruit the transcriptional coactivators GRIP1 and SRC-1 upon agonist binding." Due to this groundbreaking paper, we did not continue with some of the studies in our original hypothesis, since they were identical to the ones presented in this paper. Specifically, we intended on testing PRA or PRB and the selected coregulator for interactions using coimmunoprecipitation (co-IP) and mammalian two-hybrid screening, which was already done by Giagrande et al. (Giangrande, Kimbrel et al. 2000).

Instead of repeating this work by Giagrande, we next embarked on cellular kinetic studies of drug-receptor complexes in living cells. The hypothesis of this part of the project was to correlate the rate of import into the nucleus of drug-occupied PRB with the dose of drug and hence the transcriptional activity of the drug. The study of the nucleocytoplasmic kinetics of PR is interdisciplinary and merges pharmaceutics, pharmacology, and cellular and molecular biology. While traditional pharmaceutics methods are critically important for characterizing dose-response and drug delivery, our proposed research will be able to address unanswered questions in pharmaceutics (how long does it take for a drug or drug-receptor complex to get into the nucleus of a cell; does the rate of import correlate with gene transcriptional activity, etc.).

The cellular kinetics part of this research can be compared to pharmacodynamic studies, or "what the drug does to the body." Instead of the body, however, this research on cellular kinetics will look at what the drug (or drug-receptor complex, in this case) does to the cell at a *molecular* level. Part of the purpose of this proposal is to test the feasibility of developing cellular pharmacokinetics (is it reproducible, can the correlations between dose/response be made, etc). The knowledge of cellular kinetic studies will lead to improved treatment of steroid-receptor related diseases (such as reproductive cancers) and improved therapeutics.

Our previous paper (Lim, Baumann et al. 1999) has shown that transcriptional activity of human PRB is related to the dose of drug (R5020). The studies shown in figure 2 show that in this data set (n = 7) that the rate of import of PRB-R5020 complexes into the nucleus (can be measured by loss of cytoplasmic intensity over time) is related to the dose of drug. Roughly, if the slopes of the graphs in figure 2 are taken, the slopes (rates of import) increase as the dose of R5020 is increased.
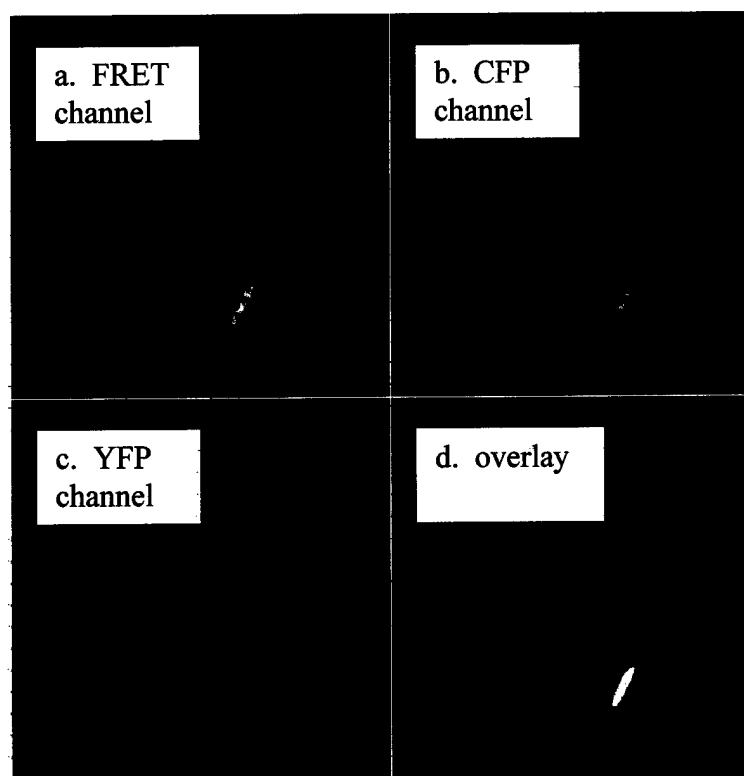
Figure 1. Fluorescence Resonance Energy Transfer (FRET) between CFP-PRB and YFP-SRC-1a. CFP-PRB shown in cyan (b); YFP-SRC-1a shown in red (c); FRET shown in yellow (a). Overlay of all 3 shown in d.
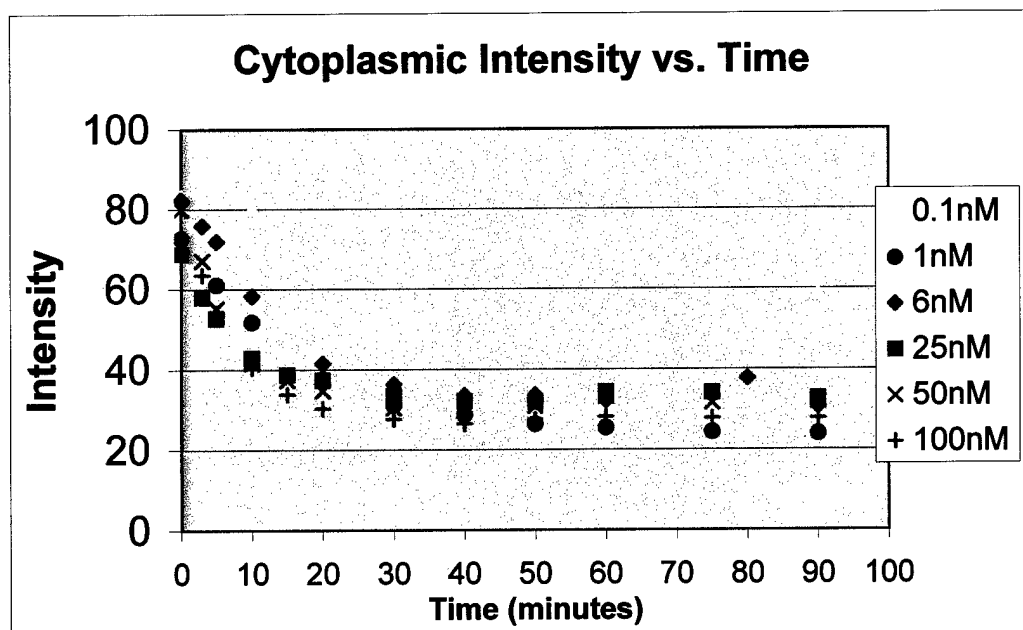

Figure 2. EGFP-PRB movement out of the cytoplasm (into the nucleus) over time, with different doses of R5020 added (0.1 to 100nM). As the dose of drug is increased, the slope of intensity vs. time curves gets steeper, indicating faster movement of R5020-occupied EGFP-PRB.

KEY RESEARCH ACCOMPLISHMENTS

-construction of plasmids pEYFP-SRC-1A, pEYFP-GRIP (GRIP is also known as TIF-2), pECFP-NCoR, pECFP-SMRT, pECFP-PRA, pEYFP-PRA, pECFP-PRB, and pEYFP-PRB.
-FRET interaction between PRB and the coactivator SRC-1 (proof of concept)
-preliminary studies indicating that rate of import of drug-occupied PRB is related to the does of drug, and therefore the transcriptional activity of PRB

REPORTABLE OUTCOMES

Abstract (to be presented as a poster at the American Association of Pharmaceutical Scientists, Nov. 2002) titled "Progesterone Receptor B-isoform in Breast Cancer Cells: Correlation of Drug Dose to Receptor Transport Rate"

Funding applied for and obtained from Pharmaceutical Research and Manufacturers of America Foundation 2002 Research Starter Grant in Pharmaceutics, $30,000/yr for 2 years, titled "Cellular Kinetics of the Delivery of Drug-Receptor Complexes to Nuclear Targets"

Funding applied for (pending; notification Jan. 2003), NIH RO1 grant, titled "Transport and Kinetics of Drug-Receptor Complexes"

CONCLUSIONS:

FRET is an exciting new technique that can be used to detect protein-protein interactions *in vivo* (Day 1998), (Day, Periasamy et al. 2001), (Xia and Liu 2001). Here we have shown (preliminarily) that the interaction between a steroid receptor, PRB, and its coactivator, SRC-1, can be detected in this manner. Future studies include further (mathematical) analysis of FRET (quantitation) for these 2 pairs (PRB and SRC-1) as well as all other pairs of potentially interacting proteins listed above (PRA/B with SRC-1, TIF-2, NCoR, and SMRT). As more steroid co-regulators are discovered, it will be important to be able to detect interactions *in vivo* that occur in the signal transduction cascade leading to reproductive cancers.

Additionally, our preliminary studies indicate that rate of import of drug-occupied PRB is related to the does of drug, and therefore the transcriptional activity of PRB. Our future work includes testing other PR agonist and antagonist drugs and determining whether the rates of import due to these drugs correlates with transcriptional activity (or transcriptional repression in the case of antagonists).

As well as being applicable to studying drug-receptor kinetics, the rate of import studies can be applied to measuring transport/trafficking of other drug transporters/receptors that have been cloned. Theoretically, any cloned protein can be tagged and traced in living cells (including human cell lines). The completion of the human genome project ensures that more new drugs will be created that work at the genetic (gene or DNA) level, involve the use of cloned recombinant protein products, or

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involve the introduction of genes encoding for proteins (gene therapy). The magnitude of these biotechnology derived products is enormous-- for protein drugs alone, sales in 1997 reached over 17 billion dollars (Putney 1999). However, drug delivery of these biotechnology derived products are currently problematic.

Consequently there will be an increasing need to track the events occurring at the cellular level; in particular, detection and tracking of entry into the nucleus and access to nuclear targets will be of great importance. As more is known about the nuclear pore complex, strategies for enhancing transport across this structure will be developed. The rate-limiting step in traditional macro-drug delivery often is the passage of the drug molecule across a barrier (skin, gut, etc). Likewise, the mechanisms governing the passage of drugs or drug-receptor complexes across the nucleus are not fully understood. It is anticipated that this type of work to have potential use in the future for the tracking of the delivery biotechnology derived products at the cellular level. Measurement of molecular processes such as these can be utilized for screening of new pharmacological agents (entry into nucleus, accumulation at nuclear targets) such as new antiprogestin birth control agents or new drugs for steroid-related reproductive cancers.

As a scientific product, the FRET studies can be used for screening interactions between newly discovered proteins involved in cancer; the cellular kinetic studies as mentioned can be used for screening pharmacological agents against reproductive cancers.

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APPENDICES
(none)